Award Number: W81XWH-12-1-0542

TITLE: Novel in Vitro Modification of Bone for an Allograft with Improved Toughness Osteoconductivity

PRINCIPAL INVESTIGATOR: Yener N. Yeni, Ph.D.

CONTRACTING ORGANIZATION: Henry Ford Health System
DETROIT MI 4822-3450

REPORT DATE: April 2014

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
April 2014	Annual	30 September 2012-29 March 2014

SUBTITLE
Novel in Vitro Modification of Bone for an Allograft with Improved Toughness Osteoconductivity

5a. CONTRACT NUMBER
W81XWH-12-1-0542

5b. GRANT NUMBER
W81XWH-12-1-0542

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Yener N. Yeni

E-Mail: yeni@bjc.hfh.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

HENRY FORD HEALTH SYSTEM
1 FORD PL ST E5F
DETROIT MI 4822-3450

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The purpose of the project is to investigate a potential application of ALT-711 (4,5-Dimethyl-3-(2-oxo-2-phenylethyl)-thiazolium chloride) in improving both mechanical and biological quality of femoral cortical bone for the purpose of providing better allograft materials via chemically breaking down AGEs (Advanced Glycation Endproducts) in the bone matrix. AGEs are naturally accumulated with age in connective tissues and have adverse effects on the biological and mechanical functions. Thus allografts sourced from bones with a high level of AGEs are likely to have a higher risk of nonunion and premature failure in the hosts. The current scope, as a proof of concept study, is to measure the effectiveness of ALT-711 on both cellular and mechanical characteristics of bones with and without prior glycation treatment (an artificial method to increase AGEs). We will measure effects of ALT-711 on osteogenic expression of stem cells on the bone substrates and also measure fracture toughness, a key measure of strong bone. We have demonstrated thus far ALT-711 has weak but measurable positive effects on the osteogenic expression of stem cells on bones without ribose treatment. We also have demonstrated ALT-711 is able to reverse the fluorescence level (assumed to be correlated to the amount of AGEs) previously increased by glycation. As such, we are encouraged by the preliminary data and now focusing our efforts in completing the rest of the tasks within the project. We expect to complete the work in the next period.

15. SUBJECT TERMS
Fracture healing, allograft bone, nonenzymatic glycation crosslinks, crosslink breaker, osteoconductivity, stem cell culture, fracture mechanics

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	23	**19b. TELEPHONE NUMBER** *(include area code)*

TABLE OF CONTENTS

INTRODUCTION

Bone grafts are used to provide mechanical support and enhance the biological repair of skeletal defects. Age-related increase of advanced glycation endproducts (AGEs) within the collagen network of skeletal tissues adversely affects the mechanical and biological qualities of the allograft tissue. It is hypothesized that treatment with an AGE-breaker compound will increase fracture resistance and osteoconductivity of an allograft. Objectives are 1) to investigate the extent to which in vitro treatment of human cortical bone with an AGE-breaker will improve the fracture toughness of the tissue by treating normal and artificially glycated tissues in different concentrations of ALT-711 (4,5-Dimethyl-3-(2-oxo-2-phenylethyl)- thiazolium chloride) solutions followed by mechanical testing; 2) to investigate the extent to which in vitro treatment of young and old femoral cortical bone with an AGE-breaker will affect the recruitment, division and osteogenic development of mesenchymal stem cells by using normal and artificially glycated demineralized bone samples (treated or non-treated with ALT-711) in a cell culture. The degree of cell proliferation will be determined by ethynyl deoxyuridine incorporation, apoptosis by Tunel technique and osteogenic differentiation by Von Kossa staining for mineral deposition, alkaline phosphatase activity and expression of bone-characteristic genes, osteocalcin, Runx2, and col1a1 by RT-PCR. High-performance liquid chromatography and fluorescence microscopy will be used to quantify AGEs and crosslinks.

BODY

The following table summarizes the current state of progress in each task (**Table 1**).

Table 1. Summary of current work status*

Tasks	Description	Progress	Status	Prerequisite
Task 1	Retrieval of 24 femurs	24 femurs acquired	Completed ✓	-
Task 2	Optimize AGE-breaker (ALT-711) treatments	Optimal ALT-711 concentrations and durations identified.	Completed ✓	-
Task 3	Preparation of test samples	24 x 4 = 96 CT test samples machined and treated. Additional beam specimens made for complementary/exploratory tests.	Completed ✓	-
Task 4	Mechanical testing	Preliminary tests performed and analyzed.	In progress	-
Task 5	Preparation of bone substrates and running cell cultures	Preparation of specimens and their treatment is complete. Preliminary test performed and analyzed. Protocol ready to be used. Pending cell cultures.	In progress	-
Task 6	Measure cell division (ethynyl deoxyuridine)	-	Not initiated yet	Task 5
Task 7	Measure apoptotic cell density (Tunel)	-	Not initiated yet	Task 5
Task 8	Measure mineral nodules (Von Kossa/histochemical)	-	Not initiated yet	Task 5
Task 9	Measure expression of osteogenic markers (alkaline phosphatase, osteocalcin, Runx2 and col2a1)	Preliminary tests performed and analyzed. Protocol ready to be used.	In progress	Task 5
Task 10	Data analysis, publications, reports	Preliminary data analysis on effects of glycation (GLY) and ALT-711 (ALT) completed.	In progress	Tasks 1-9

* See the “Problems Encountered” section.

Task 1. Retrieval of 24 cadaveric human fresh-frozen femurs from tissue banks and body donation programs. (Months 1-4.) - Completed

1a. Review and activation of tissue collection protocols by the donation program (Month 1).
1b. Collection and shipment of the femurs. (Months 1-4.)

Approval of the institutional review board (IRB 7511) for the project was obtained and submitted to the USAMRMC Office of Research Protections (ORP), Human Research Protection Office (HRPO) for the review along with a claim of exemption from the review to use preexisting cadaveric femurs. The statement that the project may proceed with no further requirement for review by the HRPO was received prior to the beginning of the supported period. In accordance with this notification (HRPO Log Number A-17384), newly retrieved femurs as well as previously existing deidentified cadaveric femurs are used.

As previously proposed, through approved tissue banks (the National Disease Research Interchange (NDRI), Platinum Training, and LifeLegacy Foundation) we obtained all required femurs meeting age, sex and medical condition criteria. The procurement of femurs took longer than initially planned as healthy donors are often prioritized for transplant surgeries. We therefore revised some of the exclusion criteria, taking into account similar work done by Wu et al. [1] and feedback from the tissue bank (**Table 2**) so as to increase the chance of procurement without sacrificing the intention of the criteria.

Table 2. Old vs. new exclusion criteria. Exclusion criteria were revised to increase the chance of procurement and reduce miscommunication with the tissue providers without sacrificing the intention of the criteria.

	Original criteria	**New criteria**
Post mortem to inventory (PMI) time	24hr	72hr
Bed rest prior to death	Ventilated state	Over 6 weeks
History of radiation or chemotherapy	Any history of radiation or chemotherapy	Last five years before death
Medication	Anticonvulsant	-
Disease/conditions	-	Osteogenesis imperfecta Cushing's syndrome Syphilis

Task 2. Optimize AGE-breaker (ALT-711) treatments (36 sections/femur; 4 distal femurs. (Months 1-4.) - Completed

2a. Preparation of thin bone sections from femurs and demineralization in EDTA solutions. (Month 1.)
2b. Artificial glycation in ribose and ALT-711 treatment of the normal and glycated subgroups (0, 0.3mM and 3mM; 0, 5, 10, 20 hour groups). (Month 1-2.)
2c. Fluorescence analysis of AGE content. (Month 2-4.)

Although the original proposal for determining the optimal ALT treatments was about demineralized bone samples to be used as substrates in Aim 2, we felt that additional experiments needed to be performed to optimize the treatment for undemineralized specimens of Aim 1. We have taken two approaches in the analysis of fluorescence, one using spectrophotometry and another using fluorescence microscopy.

i) Initial spectrophotometry experiments with powderized bone

In order to relate fluorescence readings from the spectrophotometer to a known standard, a quinine reference standard was developed. Various concentrations of quinine were prepared in 100 ml sample volumes. A linear relationship between fluorescence and concentrations of quinine was found (**Figure 1**), which would allow presentation of arbitrary units of fluorescence in equivalent ng/mL quinine.

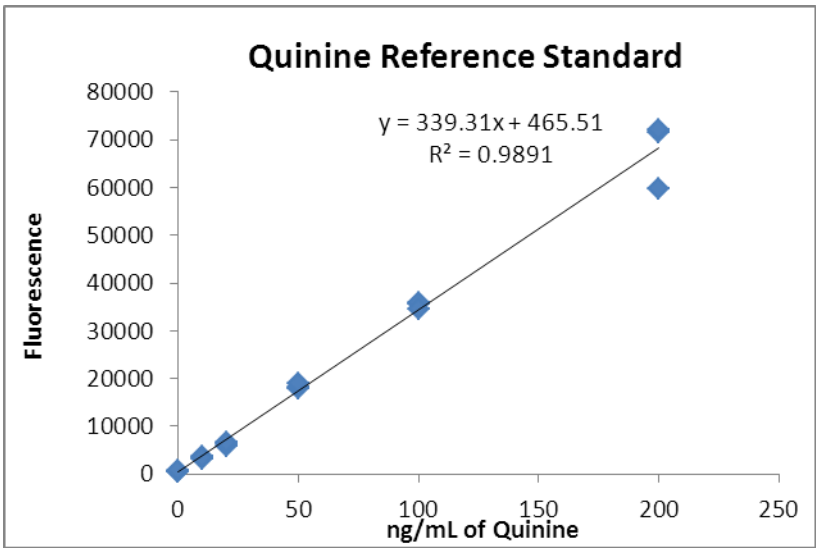

Figure 1. As expected, a strong linear relationship was established between arbitrary units of fluorescence and quinine concentrations. This relationship was intended to serve as a standard for quantification of fluorescence in our spectrophotometric approach.

For the initial studies to frame an appropriate range of bone powder concentration, ALT concentration, and treatment duration, an approximately 2x2cm section of a distal diaphysis was powdered to be used as a uniform reference. Our assumption was that the powder was sufficiently homogeneous to be used as a standard "bone" material. The decalcified specimens were digested in papain (+ Tris buffer) with 24 hours of incubation at 55 ºC and prepared into 10, 25, 50 and 100mg powders in 1ml digestion solution. In order to determine the extent to which the digestion solution fluoresces, papain + Tris buffer was also measured before and after 24 hour incubation at 55 ºC. We found that fluorescence and bone powder concentration had a linear relationship. All powder weights except 100mg were within the range of the quinine standard established above.

A slight decrease in fluorescence was observed with increasing ALT concentration (**Figure 2**). However, compared to the bone's fluorescence, the background fluorescence occurred from the buffer and ALT treatment solution was small. We concluded that the fluorescence from the digestion or ALT solution is not high enough to prevent us from measuring variations in bone. The results from a series of experiments were consistent in that 3 mM ALT at 70 hours or more duration is an effective treatment (**Figure 3**), while less than 0.3 mM is not effective and 10 mM did not increase the fluorescence (**Figure 4**). In conclusion, 3mM for about one week appears adequate but the duration of the treatment must be controlled.

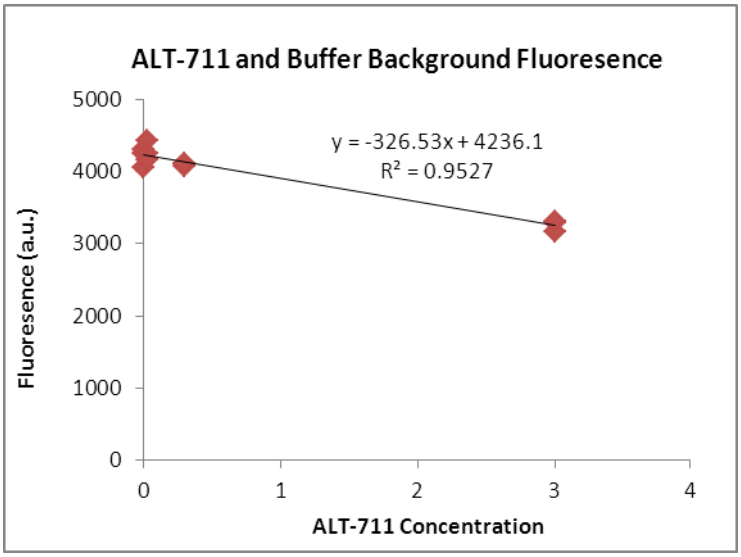

Figure 2. Fluorescence from the ALT treatment solutions at 7 days.

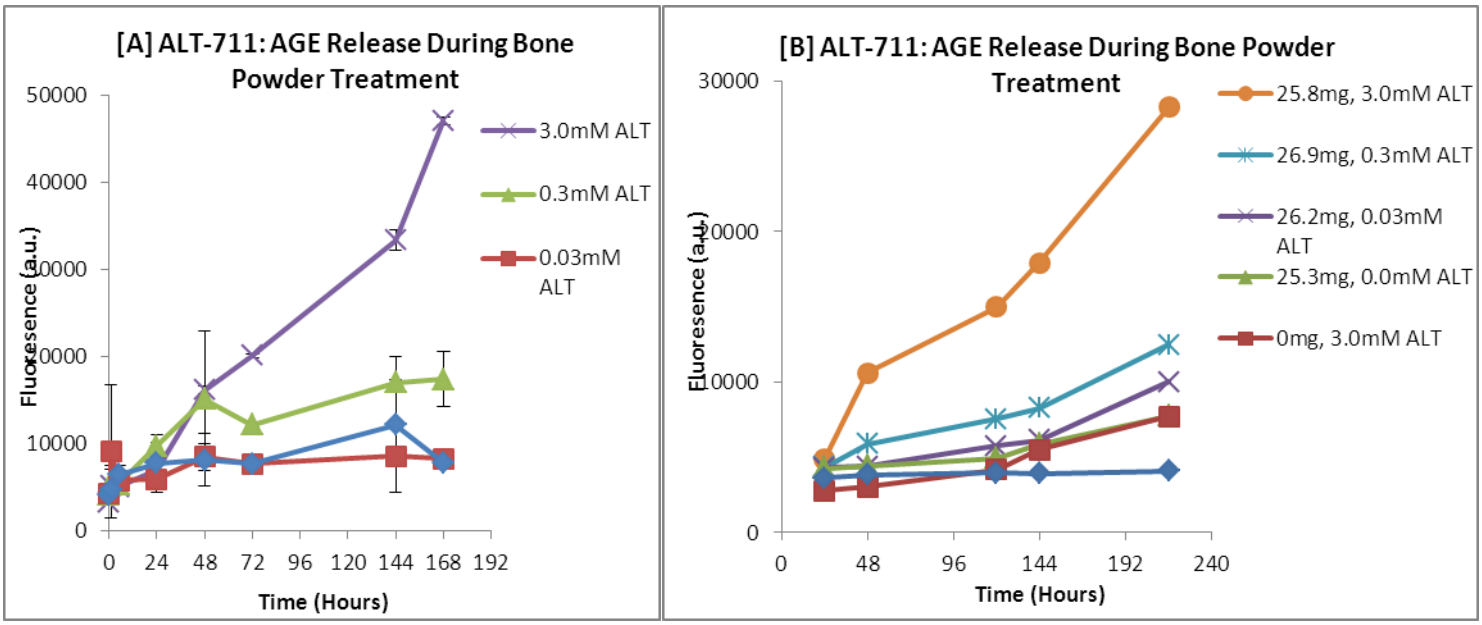

Figure 3 Results from typical series of experiments ((A) and (B)) in which the effect of time and dosage is examined. (A) These results suggest that at least 70 hours treatment in 3mM ALT is necessary to observe an effect. The error bars represent the standard deviation within triplicate measurements. (B) Another experiment using 30mg bone powder, which is in general agreement with (A) that 3 mM is effective.

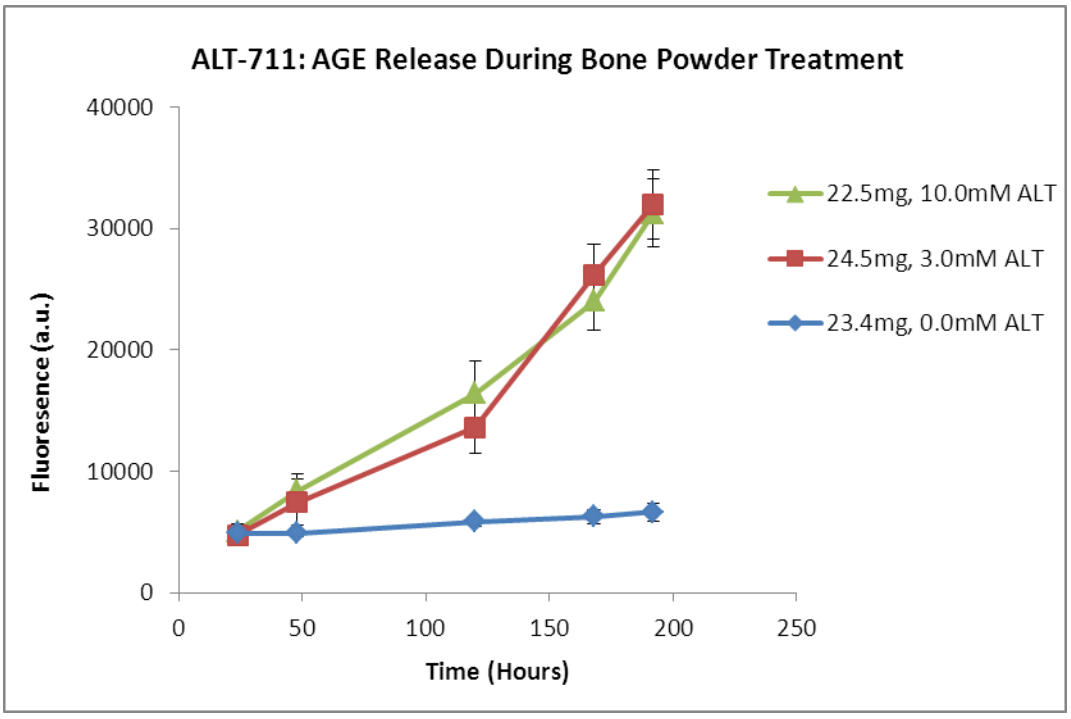

Figure 4. The experiment in **Figure 3B** was repeated with ALT concentrations of 3 mM and 10 mM to examine if the concentration of ALT was too low to be able to determine differences between the treatment concentrations. The fluorescence levels after 50 to ~200 hours were higher in treatment groups than no treatment, but the 3 mM and 10 mM groups didn't appear different.

ii) Epi-fluorescence microscopy with demineralized bone

The epi-fluorescence microscopy method has two main advantages over the spectrophotometry method in detecting relative changes in AGE concentration; i) it is a non-destructive method, and thus a longitudinal observation of changes in AGE concentrations within each sample is possible, and ii) it provides a visual distribution of AGEs across the area of examination (**Figure 5**).

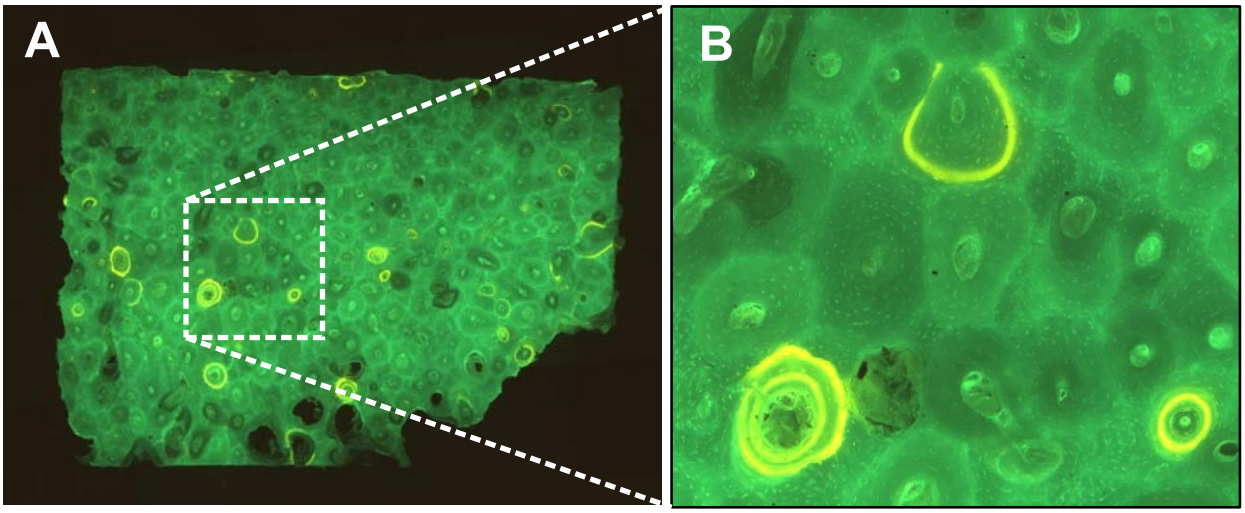

Figure 5. (A) A ~100 µm thick human femoral bone section (undecalcified) under epi-fluorescence microscopy and (B) a subregion of the section showing a magnified view of the haversian system. Osteons with lower fluorescence are younger (recently formed) while those with brighter fluorescence (due to higher AGE content) are older. Green-yellow concentric rings found around osteons are residues, possibly from antibacterial treatments (tetracycline) while the donor was still alive.

We prepared 20 femoral bone sections with thickness of 110 ± 15 µm using a circular diamond saw (South Bay, San Diego) under constant water irrigation to prevent heat damage to the tissue. A femoral bone piece (a 61 year old male donor) was aligned so that sections would reveal the axial plane (transverse to the supero-inferior direction) of the femur. The processed thin sections were further trimmed into a rectangular shape (approximately 5mm x 4mm) using a scalpel blade and a corner was also nipped to create a landmark for a reference for longitudinal imaging (**Figure 5**). The 20 sections were divided into five treatment groups (**Table 3**) and PBS buffer together with chloroform (20 µL/25 mL) and gentamicin (25 µL/25 mL) to inhibit fungal and bacterial growth during 33 days of incubation (37 °C).

Before EDTA decalcification, they were individually wet mounted and photographed under epi-fluorescence microscopy (Nikon Corp., Tokyo, Japan) using a x4 objective with 60 ms exposure time. The light source was ultraviolet (UV) through 400±440nm excitation and 480nm barrier filters. Sections were decalcified for 50 hours and then another set of images were taken for day 0 reference. Longitudinal fluorescence imaging was carried out every 2-3 days up to two weeks and then weekly thereafter up to 33rd day (**Figure 6**).

Table 3. Description of the treatment groups. Each treatment group included 4 sections.

Treatment group notation	**Followup treatment**	**Description**	**Treatment solution**	**Storage temp**	**Duration**
GLY	GLY → ALT(n=2)	Initially glycated only but later divided into two groups GLY→ALT and GLY→PBS at day 22.	Ribose (666 mM) ALT-711 (3 mM) PBS	37 °C	GLY = 22 days ALT = 11 days PBS = 11 days
	GLY → PBS(n=2)				
GLY+ALT		Glycated for 7 days and then ALT	Ribose (666 mM) ALT-711 (3 mM)	37 °C	GLY = 7 days ALT = 26 days
ALT		ALT-711 only	ALT-711 (3 mM)	20 °C	33 days
C		Control – no treatment	PBS	20 °C	33 days
F		Frozen control – no treatment and kept in a freezer	PBS	-20 °C	33 days

We observed a rapid increase in fluorescence levels in glycated specimens (**Figures 6-7**), however when ALT-711 was introduced in the GLY+ALT group at day 7, the increase in FL from the previous glycation treatment was reversed, reducing FL by 15%. However, after day 13 a slow but steady increase in FL could be observed again. In GLY specimens, ALT-711 treatment was introduced to 2 of 4 GLY sections at day 22 while the other 2 sections were placed in PBS solution to confirm the ALT's effect in reducing FL observed in the GLY+ALT group between day 7 and 13. At this point, all four GLY sections were at a nearly saturated state from 22 days of GLY treatment. Upon ALT-711 treament on the GLY saturatrated sections, the FL decreased at day 27 and then further decreased up on a replenishment of ALT-711 solution at day 29, resulting in a total decrease of 14%.

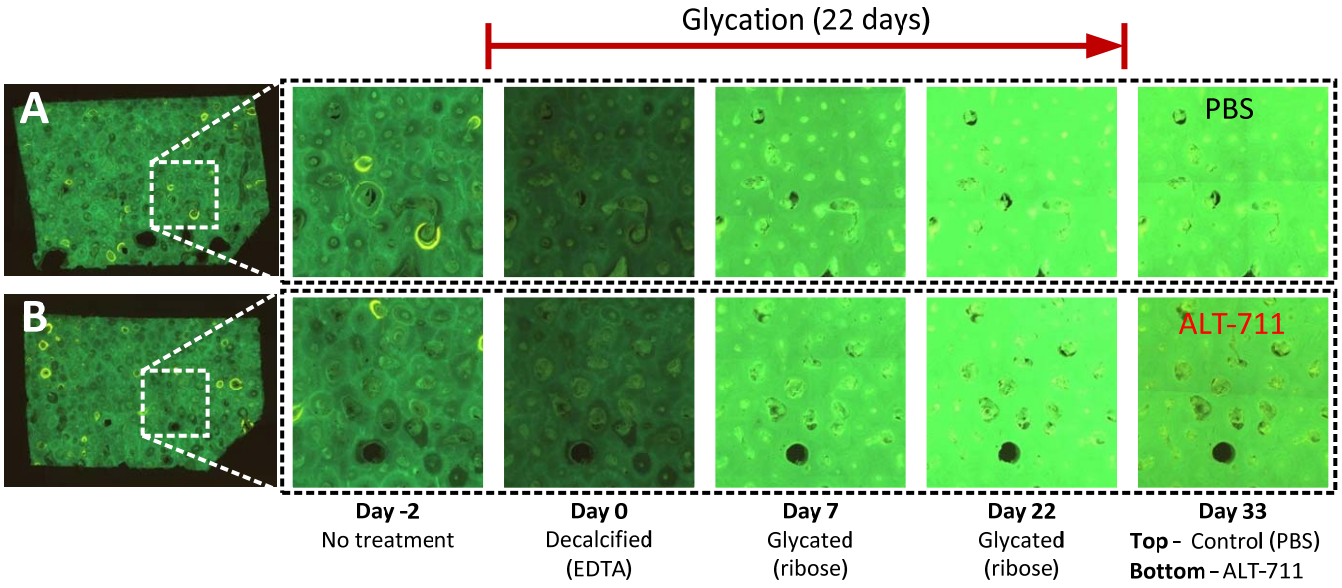

Figure 6. Longitudinal changes of fluorescence level (FL) from decalcification, glycation and ALT-711 treatments. Both (A) and (B) show steady increase of FL during glycation up to 22nd day. The FL is lower after 11 days of ALT-711 treatment (Day 33) than at 22nd day of glycation (B) while no clear difference was observed after 11 days of PBS treatment (control) following the 22 day glycation regimen (A).

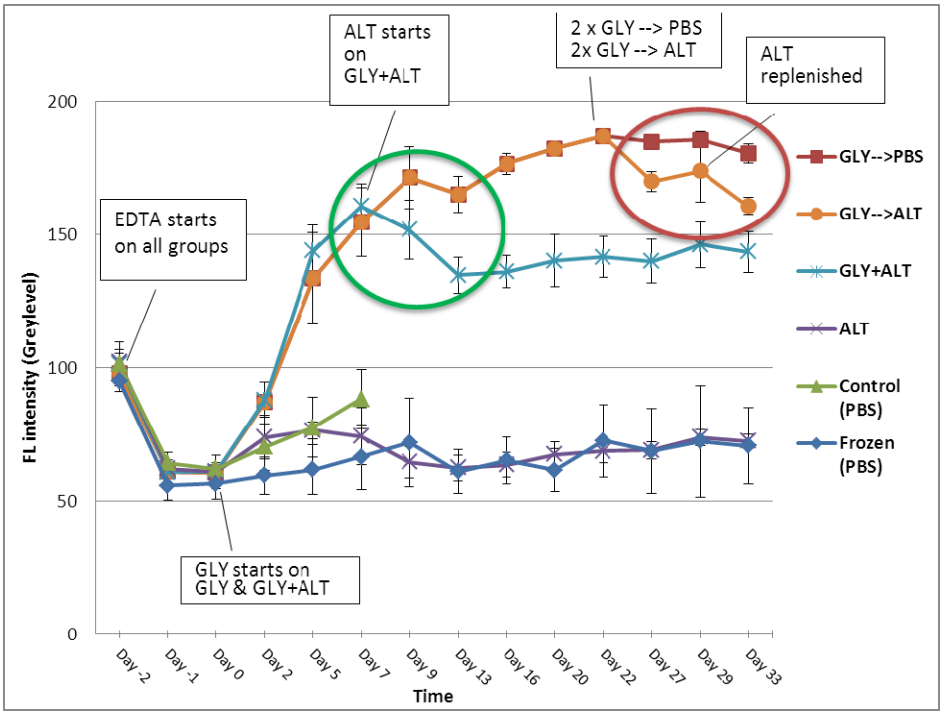

Figure 7. Longitudinal changes in average fluorescence level (FL) for each group during decalcification, glycation and ALT-711 treatments. A marked drop in FL can be observed for the GLY+ALT group during the period from day 7 to 13 (green circle) and for the GLY→ALT group during the period from day 22 to 33 (red circle). Error bars indicate standard deviations.

The ALT group, where ALT-711 was immediately applied to freshly decalcified sections, did not show a difference from the control groups.

In order to confirm the ability of ALT-711 to reverse the increased FL by ribose glycation, we conducted another similar experiment with 40 bone sections from a single donor, allocating 10 sections to each group (i.e. GLY→PBS, GLY→ALT, ALT, PBS control). In this experiment all four groups were subjected to a 37 °C incubation condition as opposed to the previous experiment, in which the ALT and control groups were kept at room temperature. As in the previous experiment, we observed a marked decrease (-20%) in FL levels that were initially raised by glycation (GLY→ALT, $p < 0.0048$) relative to the group that was treated with PBS after glycation (GLY→ PBS; control). Additionally, no substantial differences were observed in the ALT and control groups over the 14 day period (**Figure 8**).

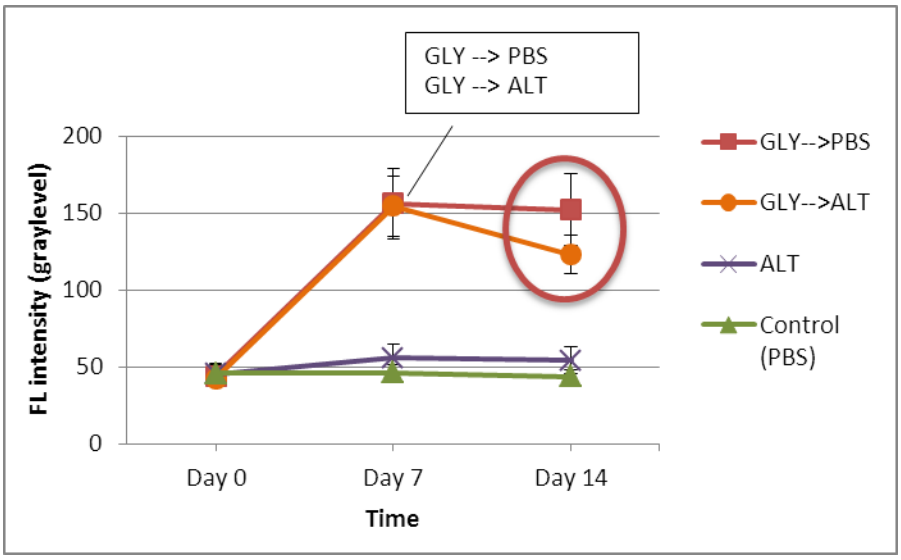

Figure 8. Both GLY→PBS and GLY→ALT groups have identically increased their FL at day 7 by glycation and there is a subsequent decrease from ALT-711 in GLY→ALT while no significant change is seen in GLY→PBS. Both ALT and PBS control groups did not change their FL significantly over the 14 day period. This experiment confirms that ALT-711 has a significant reducing effect on FL in previously glycated samples.

The results of both experiments suggest that ALT-711 of 3 mMol concentration over 7 day application has a significant effect on reducing the level of fluorescence intensity (i.e., AGE content) previously increased by ribose glycation ($p < 0.0048$). However, after day 7, its potency is reduced and further replenishment of the solution was needed. This suggests that 3 mM of ALT-711 has a potent duration of approximately 7 days but its effect is cumulative and can be continued by replenishing the solution. Based on the projection on the FL gradients in **Figure 7** and **Figure 8**, over 30 days of ALT-711 treatment may reverse a significant amount of FL gained by the glycation process.

iii) Undemineralized bone

Stability of pH over incubation period

Ribose-glycation solutions are known to become acidic over time [3, 4], which can lead to unwanted decalcification of bone specimens during a long incubation period. It is not known if ALT solutions exhibit similar behavior. In order to investigated whether ribose or ALT-711 solutions would maintain a neutral pH level (i.e. 7) over a 14 day incubation period at 37 °C, we prepared 25 ml solutions (**Table 4**) with combinations of PBS, ribose (666 mM), ALT-711 (3 mM) and $CaCl_2$ (57.5 mg/liter) [2].

There was only a minor decrease of pH level in ALT-711 solutions (**Table 4, Figure 9**). Unlike unbuffered saline solutions, addition of $CaCl_2$ to PBS solutions ALT resulted in a precipitate (potentially calcium phosphate), but this caused no discernible change to the pH. Based on these observations, we decided to use ALT solutions without additional buffers for the anticipated treatment durations. On the other hand, we found a marked decrease in pH (from 6.91 to 4.32) for all ribose solutions by the end of day 14 (**Table 4, Figure 9**), confirming the need to control pH in glycation solutions. By performing a daily titration of the solution with 0.1M

NaOH [3, 4], we were able to maintain the pH of the ribose solutions at 7.2 ± 0.4 (mean ± SD) throughout the 14 day period. Based on this result, we decided to continue with daily titration and weekly replenishment of the ribose solutions for the rest of the experiments.

Table 4. Description of the solutions and pH values over a 2 week period of incubation at 37 °C. The three ribose containing treatment groups (GLY, GLY+CaCl2, GLY+ALT) are substantially acidified in 14 days (marked by *) decreasing their pH from 7 to 4.32.

			pH measured		
Treatment group	n	Treatment solution	Day 0	Day 7	Day 14
Control	1	PBS	7.01	6.99	7.02
$CaCl_2$ control	1	PBS+$CaCl_2$	7.01	6.96	6.99
ALT	1	ALT-711+PBS	7.01	6.85	6.72
ALT+ $CaCl_2$	1	ALT-711+PBS+$CaCl_2$	7.01	6.87	6.74
GLY	2	Ribose+PBS	6.86	5.28	4.32*
GLY+ $CaCl_2$	1	Ribose+PBS+$CaCl_2$	6.82	5.42	4.35*
GLY+ALT	1	Ribose+ALT-711+PBS+$CaCl_2$	6.91	5.63	4.47*

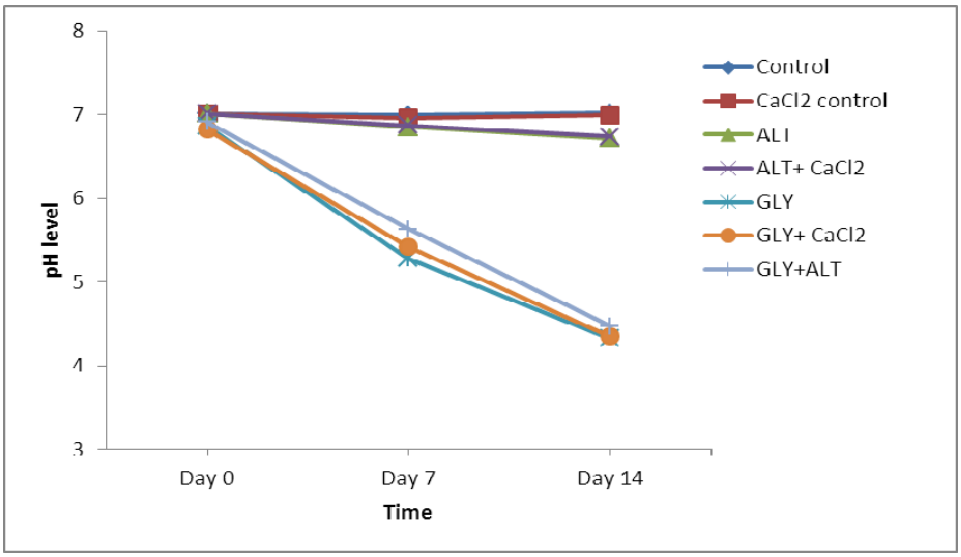

Figure 9. pH level change during a 14 day incubation period (37 °C) of ALT-711 (ALT) and ribose (GLY) solutions with and without $CaCl_2$. Any treatment solutions containing ribose (GLY) acidified quickly turning into a mild acid in two weeks.

In order to further refine and validate the dosage and duration of ALT treatment for undemineralized bone samples to be used in the mechanical tests, 2x2x5 mm beams (prepared from the region shown in **Figure 10**) were utilized. Beam dimensions were chosen to reflect the thickness of compact tension specimens (Task 3). Using a diamond circular saw (South Bay, San Diego), 36 beams were prepared from four donors (35 year old female, 50 year old male, 80 year old female and 81 year old male) representative of the gender and age groups of the main experiment.

The 36 beams were randomized and allocated into 3 x 4 wells (**Figure 10**), corresponding to three treatment groups (low ALT (3mM), high ALT (30mM) and control groups) and four time points (day 0, 7, 14, 21). All beams were initially subjected to a 2-week ribose glycation treatment (666 mM) at 37 °C. After the glycation treatment, ALT treated groups were subjected to 3 mM and 30 mM ALT solution for 7, 14 and 21 days (**Figure 5**) while the control was placed in a PBS solution.

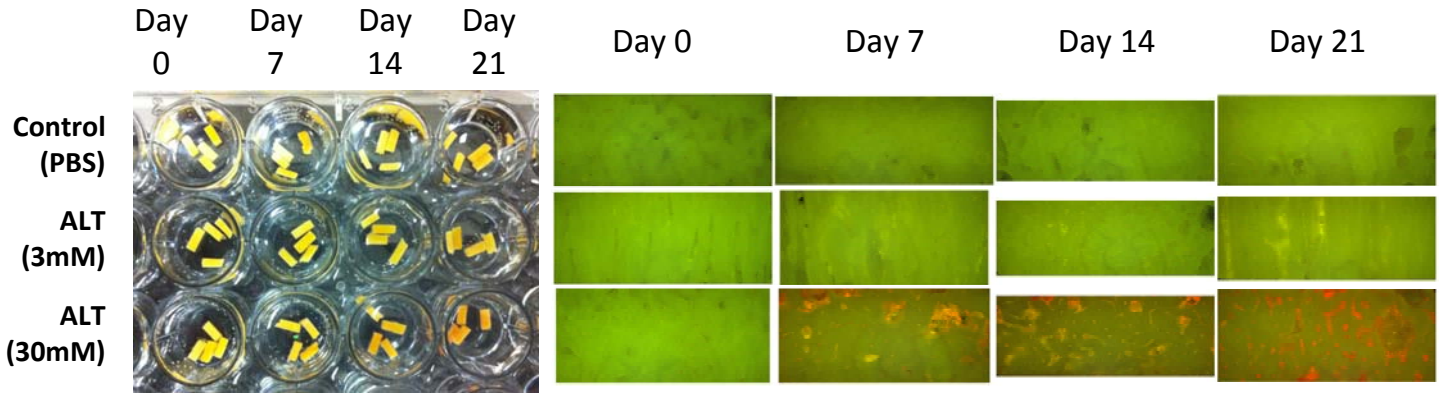

Figure 10. (Left) Bone beams allocated into 24-well plate in a 3 x 4 scheme representing three treatment groups (PBS, 3mM and 30mM ALT and four different time points from day 0 to day 21). (Right) Bone beams imaged using epi-fluorescence microscopy. The brightness of green fluorescence, a marker for glycation, decreased markedly with time in the 30mM ALT group compared to the 3mM ALT group and control, indicating ALT potentially reduces the amount of AGE crosslinks in the tissue.

The beams were processed similar to the methods of the bone powder experiment. The bone beams were first decalcified using 20% formic acid and then digested using papain solution (0.4 mg/mL in sodium acetate buffer) at 65°C for two days. The partially digested beams were crushed and further digested another two days without replacing the solution, and then specimens were centrifuged for 30 minutes at 11,000 rpm at 4°C. 100 µl of the supernatant from each specimen was collected and analyzed using spectrophotometry. According to a two-way ANOVA, both the concentration and duration significantly affected the level of fluorescence ($p<0.003$ and $p<0.0001$, respectively); the level of fluorescence generally increased with concentration and duration of ALT treatment. Post-hoc analyses using Tukey's test revealed that the significant effect of ALT (compared to PBS control) was observable at day 21 between the groups, but not before (**Figure 11**).

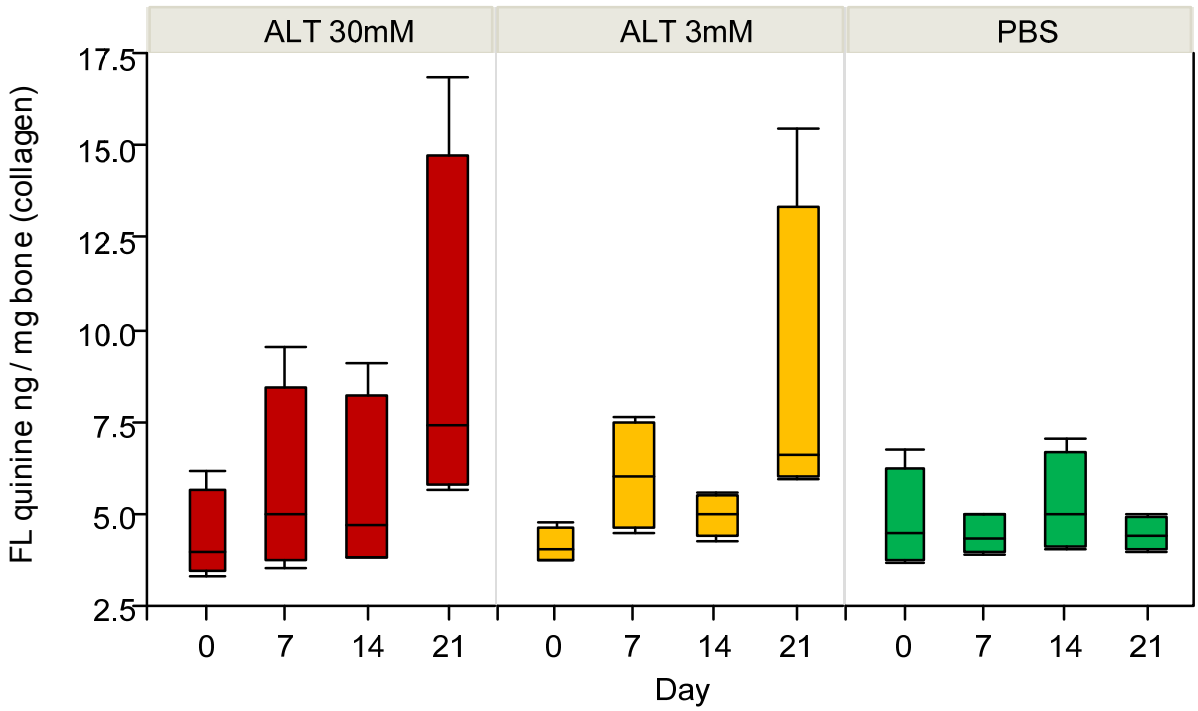

Figure 11. The amount of fluorescence under two ALT dosages (3 and 30 mM) over 21 days of treatment. An increase is observed in fluorescence level (FL) with increasing treatment duration for both 30 mM and 3 mM ALT dosages, with differences becoming significant at day 21. No change in FL was observed with increasing duration for the control (PBS).

Task 3. Preparation of test samples from 24 femurs. (Months 2-8.)
3a. Machining of 4 compact tension test specimens per femur using a CNC micromilling machine. (Months 2-6)
3b. Artificial glycation in ribose and AGE-breaker treatment of the normal and glycated subgroups (Months 7-8)

We have used a CNC milling machine (Denford Micromill 2000, West Yorkshire, UK) to machine compact tension (CT) specimens based on our previous work [5, 6]. While previous work used a saline drip irrigation method to prevent over heating of bone during machining, we machined the bone specimens submerged in a saline bath to further minimize the potential effects of heating and drying that may inadvertently degrade the bone quality (**Figure 12**).

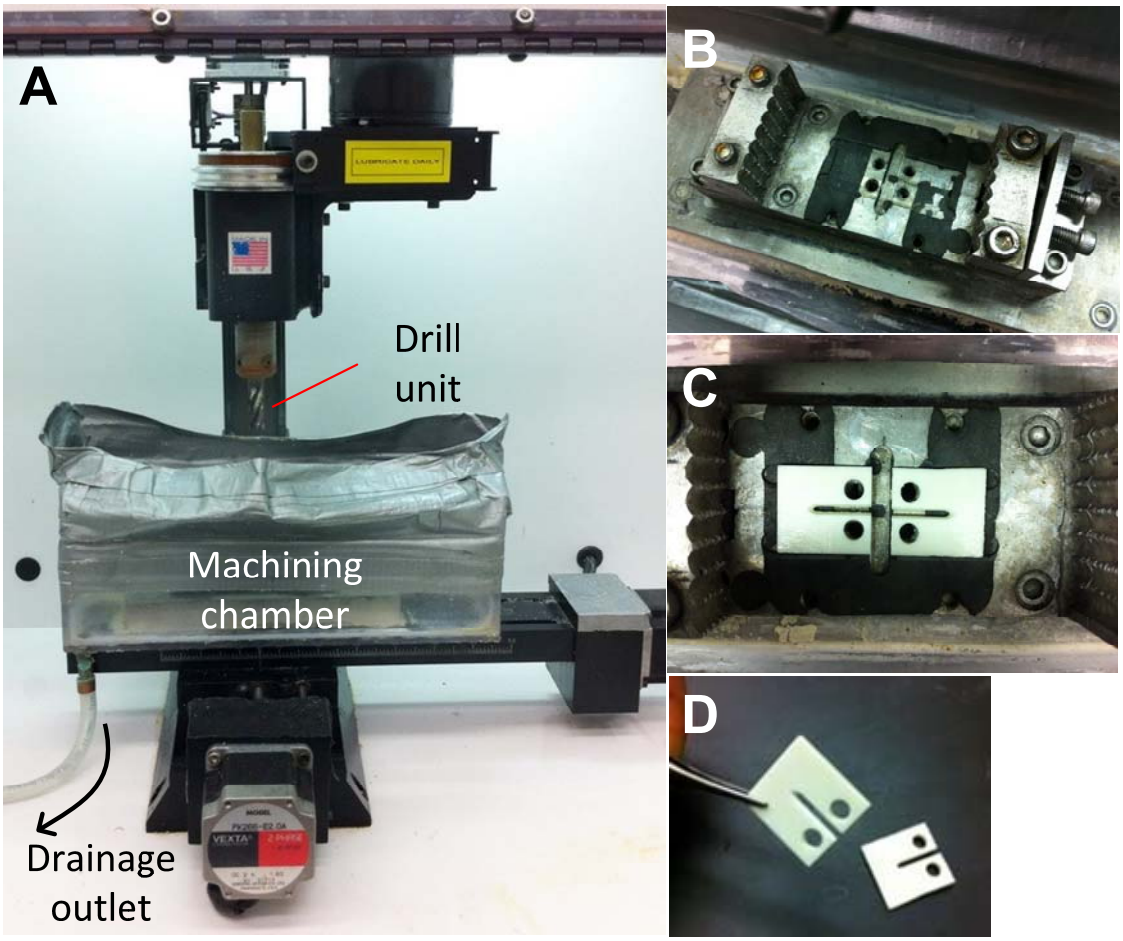

Figure 12. (A) CNC setup for compact tension (CT) specimen machining where the machining chamber was filled with saline during the entire machining process. The chamber contains (B) a specimen vice to grip the femoral section during the initial cutting process and (C) a die platform to hold the CT specimens during machining of the finer details. (D) Typical fully machined CT specimens.

Machining CT specimens fully submerged underwater required remanufacturing of the CNC machining station to incorporate a saline bath chamber, a mini vice and a drainage system. Furthermore, the CNC machining program was rewritten to account for special restrictions from machining underwater.

From each femur, we sought to obtain as many samples as possible (**Figure 13**). 4 CT specimens were obtained from medial-proximal and lateral-proximal facets while the remaining bone was used to prepare 2x2 mm beams for complementary mechanical tests and 100-200 μm thick bone slices for Aim 2. We have processed 24 femurs in this manner obtaining 96 CT samples (24 donors x 4 treatments), which were scanned on a flatbed scanner to examine the symmetricity of the chevron notch. Using an image analysis software, tip-to-edge distances (**Figure 14**) were measured and used for calculating the offset distance of the notch tip from the center of the speciemen. The notch tip to center distance was 0.04 ± 0.39 mm (mean ± SD) for the whole group of 96 CT samples.

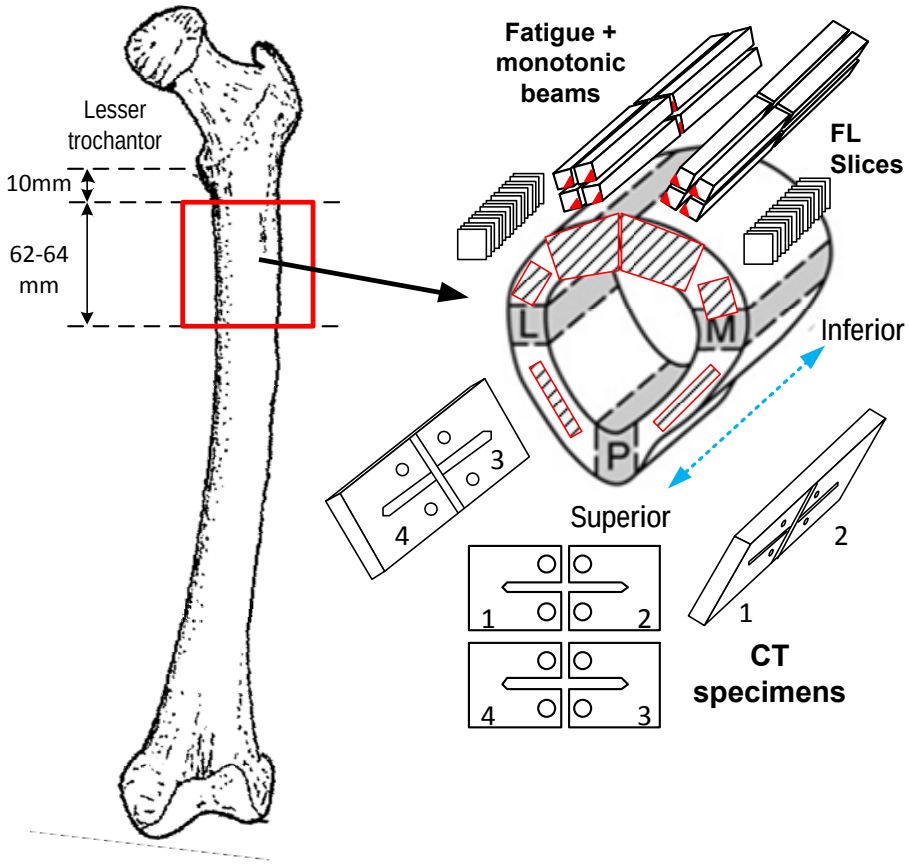

Figure 13. Cutting scheme of a femur. A 62-64 mm section was cut 10 mm below the lesser trochanter from each femur. The section was divided between medial and lateral segments and then CNC machined to produce four CT specimens from the proximal-medial and proximal-lateral cortices. The remaining bone pieces were used to cut fatigue/monotonic test beams and 100 μm thick bone slices for the osteogenicity experiments.

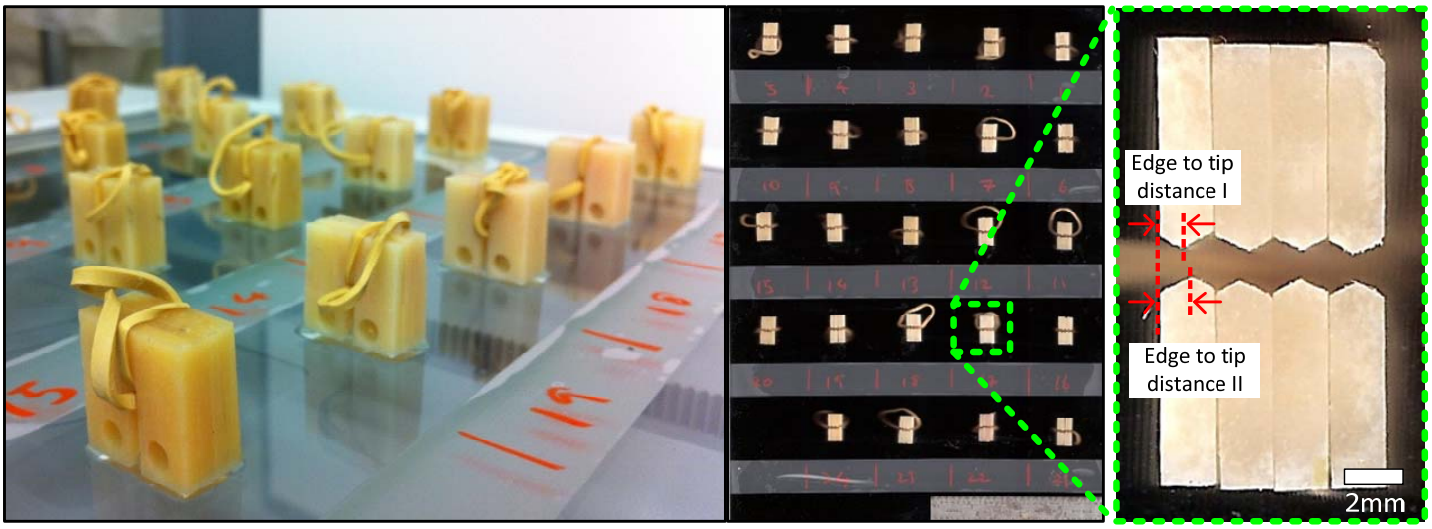

Figure 14. Four CT specimens from each donor were bound together by a rubber band and then scanned on a flatbed scanner to examine the chevron notch profile. Upper and lower edge-to-tip distances were measured and used to calculate the offset of each notch tip from the center of the specimen.

Refinement of R-curve test methods for measurement of crack resistance in bone specimens

In order to initiate controlled crack propagation, a pre-crack with 0.5mm length was made at the tip of the chevron notch in the CT specimens by using a mechanical testing system (Biosyntech Mach 1) with a razor blade attachment (**Figure 15**). The precracking procedure incorporated a contact-load detection function to accurately define the initial point of contact between the edge of the razor blade and the notch tip. To optimize the cyclic loading parameters for the fracture toughness test in the same mechanical testing system (**Figure 16**), we used six extra CT specimens. After several trials, we found that a tensile displacement amplitude of 50μm, followed by an immediate recovery of 30μm at a rate of 5μm/s provided more than six cycles of crack propagation prior to the sudden drop of tensile force, which allows for sufficient number of compliance calculations necessary for the construction of an R-curve. During the entire loading and unloading cycle, the sample was placed in an environmental chamber with constant saline irrigation.

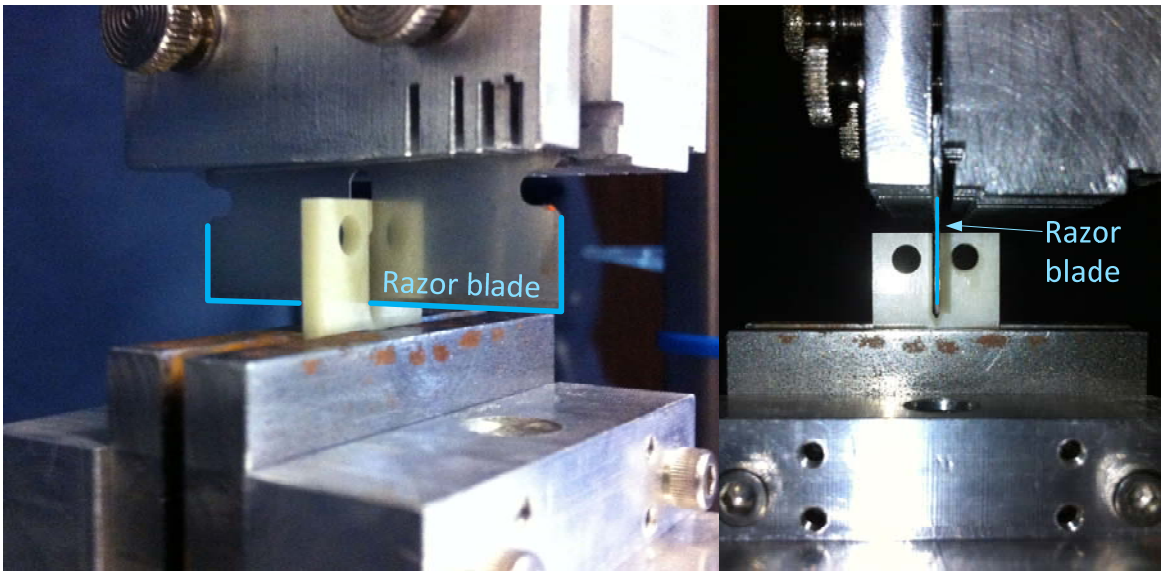

Figure 15. Prior to CT fracture toughness test, a pre-crack with a depth of 0.5mm was introduced at the tip of chevron notch using a razor blade (marked by the blue lines).

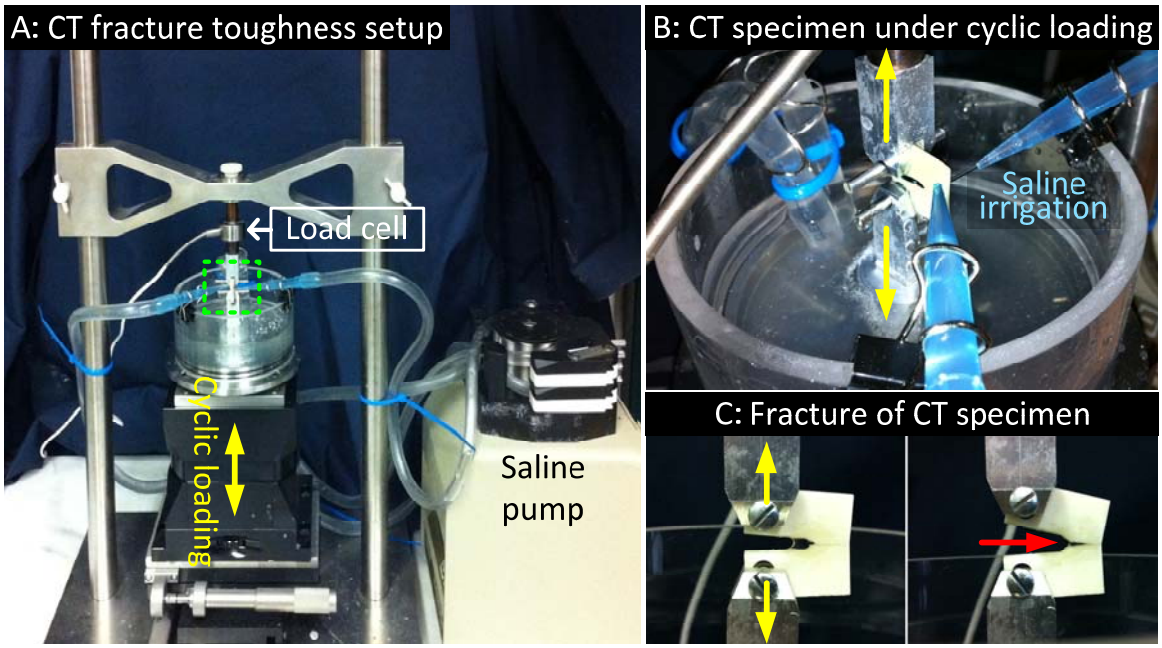

Figure 16. [A] CT specimen was cyclically loaded by a micromechanical tester with [B] constant saline irrigation. [C] Controlled propagation of a crack is achieved in the CT specimen (red arrow) upon continuous cyclic loading (yellow arrows). The purpose of the test is to determine the resistance of bone to the growth of this crack.

Fracture toughness (K_R) was calculated based on the following equation [7]:

$$K_R = \frac{PY(a/W)}{B\sqrt{W}}$$

where **P** is the maximum load associated with a crack advancement, **B** is specimen thickness, **W** is specimen width, **a** is crack length and **Y(a/W)** is a function of specimen geometry (**Figure 17**). This completes our efforts to finalize the fracture testing protocol. With the specimens machined and treated according to their experimental group assignment (glycated, ALT-treated, both glycated and ALT-treated or untreated control), we are ready to conduct the fracture tests.

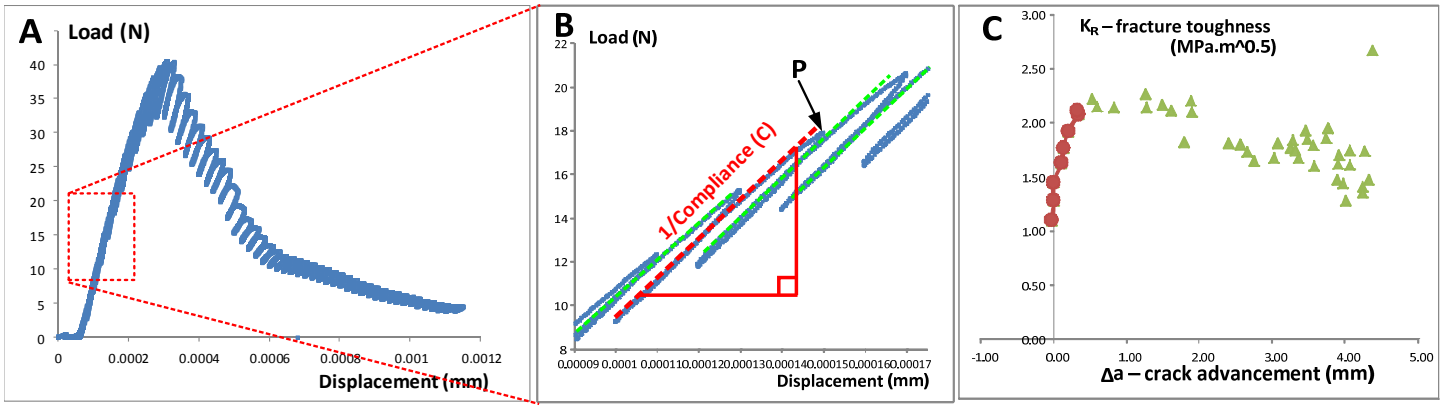

Figure 17. [A] Load-displacement curve from a typical R-curve test conducted on a bone specimen, showing series of loading-unloading cycles. [B] For each loading-unloading cycle, compliance (C) and P (maximum load) are measured. The series of compliance values are used to calculate a series of crack length values. [C] The load and crack length data are then used to construct an R-curve where fracture toughness (K_R) is plot as a function of increases in crack length (Δa) [8-10]. The rising portion of the R-curve (red) represents increasing resistance to crack propagation whereas the falling portion (green) signifies a decline in the resistance.

Task 5. Preparation of bone substrates for cell cultures from 24 femurs. (Months 4-7.)

Task 5a-c - Completed

5a. Preparation of 100 µm-thick sections (triplicates per measurement) from the middiaphysis. (Months 4-5.)
5b. Demineralization of bone sections in EDTA solutions. (Months 5-6.)
5c. Artificial glycation in ribose and AGE-breaker treatment of the normal and glycated subgroups. (Months 6-7.)

While machining CT and beam specimens from each femur for Aim 1, we also prepared approximately 500 100-200 µm thick bone slices (**Figures 13**) under this task. The bone slice substrates were then divided into four treatment groups (Glycation, Glycation + ALT-711, ALT-711 and control, **Figure 18**) and according to the treatment group, treated for one week under glycation (666 mM ribose) and/or two weeks under ALT (30mM ALT-711) consistent with the CT specimen treatment regime.

All specimens necessary for cell cultures are ready.

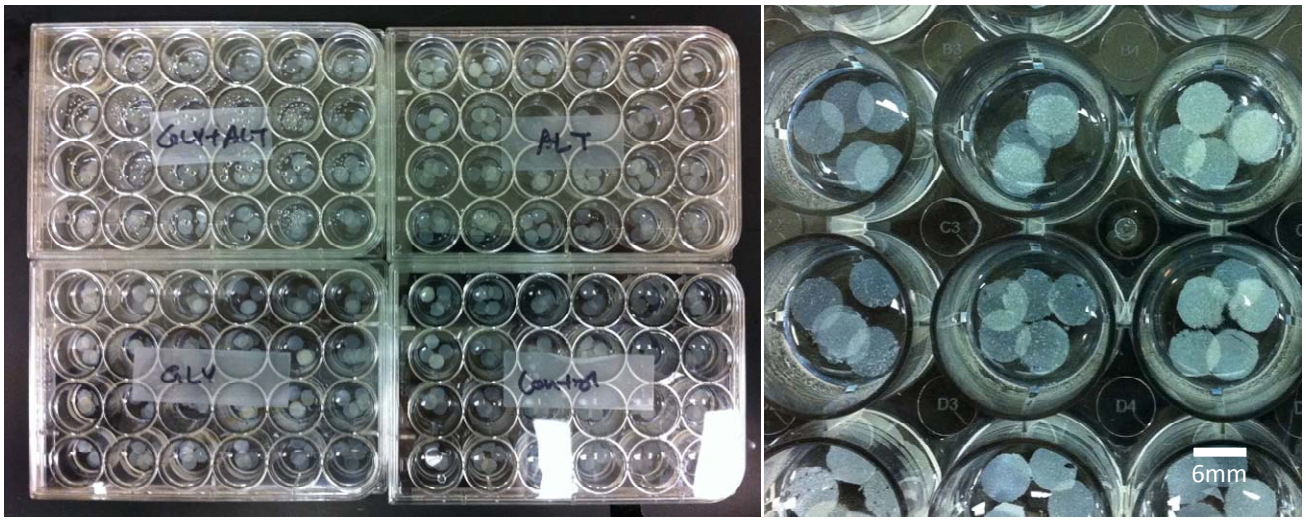

Figure 18. Approximately 500 bone slices of 6mm diameter (by using a 6mm diameter biopsy punch) were obtained from 24 femurs and divided into four treatment groups (Glycation, Glycation + ALT-711, ALT-711 and control).

Tasks 5d through Task 9.

5d. Culture human mesenchymal stem cells (Lonza, Walkersville, MD) on bone sections. (Months 7-14.) Subsequently, measure cell division (Task 6), apoptosis (Task 7), mineralization (Task 8) and expression of molecular markers of mineralization, osteocalcin, Runx2 and col1a1 using quantitative RT-PCR with specific primers. (Months 8-15.)

The purpose of this preliminary investigation was two-fold;

1) to verify whether the current proposed culture protocol was able to induce osteogenic differentiation in the stem cells on treated bone substrates,

2) to examine whether there is a measurable improvement in osteogenic expression markers by ALT-711 treatment in bone sections that has naturally accumulated AGEs (i.e. control-PBS vs ALT) and in bone sections with artificially accumulated AGEs (i.e. GLY vs GLY+ALT).

We have measured four gene expression markers (**Table 5**) that indicate the activity and stage of cell differentiation and then compared with the known temporal gene expression patterns from the literature. And then using those relative levels of gene expression markers, we statistically compared between four groups; i) ALT vs PBS and ii) GLY+ALT vs GLY at day 7 and 14.

Table 5. Summary of osteogenic markers used in this study.

Osteogenic marker	Description
Alkaline phosphatase (ALPH)	Associated with early cellular activity and differentiation but not bone specific
Collagen, type I, alpha 1 (COL1A1)	Associated with cell adhesion, proliferation and differentiation of the osteoblast phenotype and known as an early indicator of osteoblastic differentiation
RUNX2 (RUNX2)	Associated with osteoblastic differentiation and skeletal development
Osteocalcin (OCN)	Osteoblast specific protein, used as a marker for bone formation process (matrix synthesis and mineralization)

The 40 bone sections previously treated with ALT(14 days), GLY(14d), GLY(7d) +ALT(7d) and PBS(14d) (as described in Task 2 in **Figure 6**) were used as substrates for human stem cells (Lonza, Walkersville, MD). Prior to cell culture, the bone sections were washed with ethanol, hood-dried for 30 minutes and then washed again using PBS. The sections were kept in 10% bovine fetal serum DMEM solution mix with Fungizone overnight.

Table 6. RT-PCR results (mean and standard deviation) after 7 and 14 day culture showing fold change relative to corresponding PBS control group at day 7 and 14 respectively. The results are shown in **Figure 19** and **Figure 21** as scatter and box plots. We did not measure osteocalcin at day 7 because osteocalcin does not express at early stages of differentiation.

		ALT		GLY		GLY+ALT		PBS	
		Day 7	Day 14	Day 7	Day 14	Day 7	Day 14	Day 7	Day 14
ALPH	Mean	0.863	1.484	1.784	2.501	1.592	2.358	1.038	1.032
	Std Dev	0.160	0.324	0.066	0.271	0.147	0.516	0.272	0.273
COL1A1	Mean	1.333	0.825	0.681	0.549	0.561	0.585	1.050	1.059
	Std Dev	0.370	0.155	0.101	0.226	0.127	0.086	0.370	0.353
RUNX2	Mean	1.112	0.973	1.001	1.416	0.935	1.170	1.012	1.042
	Std Dev	0.136	0.191	0.043	0.261	0.128	0.203	0.165	0.288
OCN	Mean	-	0.831	-	1.083	-	0.908	-	1.115
	Std Dev	-	0.574	-	0.206	-	0.434	-	0.653

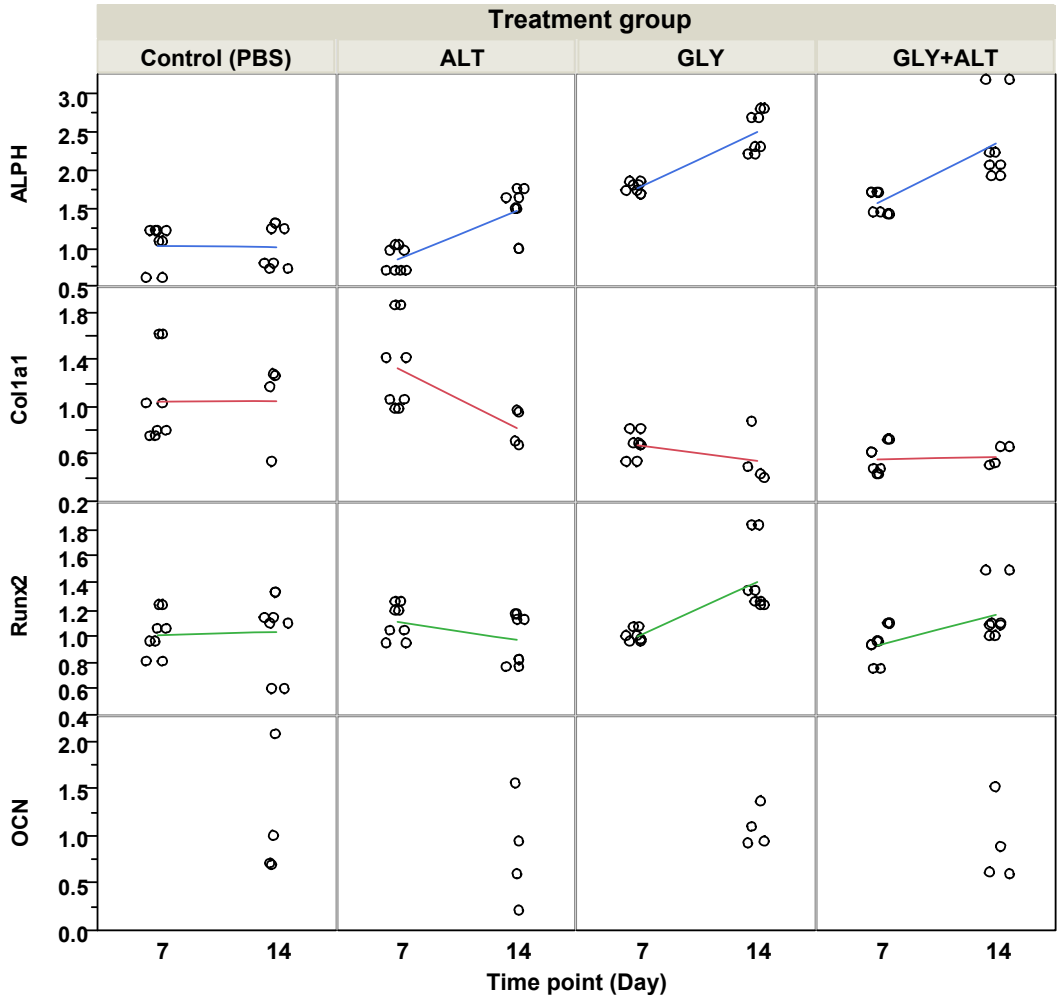

Figure 19. Temporal fold change of osteogenic markers in ALT, GLY, GLY+ALT and PBS groups at day 7 and day 14 of osteogenic culture environment. The four osteogenic markers are alkaline phosphatase (ALPH - blue), collagen 1a1 (Col1a1 - red), Runx-2 (green) and osteocalcin (OCN). The increase in ALPH and Runx2 and decrease in Col1a1 from day 7 to 14 are hallmark signs of osteoblastic differentiation. We did not measure osteocalcin at day 7 (but we did at day 14) because osteocalcin levels are undetectable in immature osteoblastic cells. Gene expression was measured, normalized to constitutive gene expression (b-actin) and expressed relative to that of PBS treated bone sections respective of the time point (day 7 or day 14 of culture respectively).

The bone sections were placed in 6 well culture plates, seeded with the stem cells and cultured under conditions shown to induce osteogenic differentiation (minimal essential medium supplemented with 10% fetal calf serum, 0.1 µM dexamethasone, 10 nM β-glycerol phosphate, and 50 µg/ml ascorbic acid phosphate). Osteogenic cell differentiation was examined by the relative expression of four osteogenic/cellular markers (i.e., alkaline phosphatase, collagen-1a1, Runx-2, osteocalcin).

Between day 7 and day 14 of cell culture, all three treatment groups (i.e., ALT, GLY and GLY+ALT) relative to the control group (PBS) have upregulated alkaline phosphatase (ALPH) while only GLY and GLY+ALT have upregulated Runx2 (**Table 6, Figure 19**). The GLY showed the highest average fold change at day 14 in both ALPH and Runx2. In col1a1 expression, ALT showed the highest rate of decrease while GLY and GLY+ALT showed significantly lower regulation of Col1a1 than the control ($p = 0.0013$ to $p = 0.0123$). We found no difference in osteocalcin expression among the groups ($p = 0.4345$ to $p = 0.9289$).

The data show that bone slices induce osteogenic differentiation of stem cells relative to that seen on plastic and that the high levels of AGE products generated by ribose treatment can further stimulate osteogenesis as measured by ALPH and Runx2 expression. The current results are largely consistent with the temporal osteogenic gene expression in osteoblasts [11, 12] where upregulation of ALPH and Runx2 and downregulations for Col1 is a hallmark of known temporal expression behavior of osteoprogenitor to pre-osteoblasts (**Figure 20**). Also the absence in detectable changes in osteocalcin expression suggests that none of the groups have yet matured beyond pre-osteoblastic stage, which is expected to occur around day 21. In conclusion, our data suggest that the current osteogenic culture was able to induce normal osteogenic differentiation in the stem cells and thus any differences in osteogenic expression between the ALT-711 treated groups and other treatment groups will likely to be due to the treatment factor.

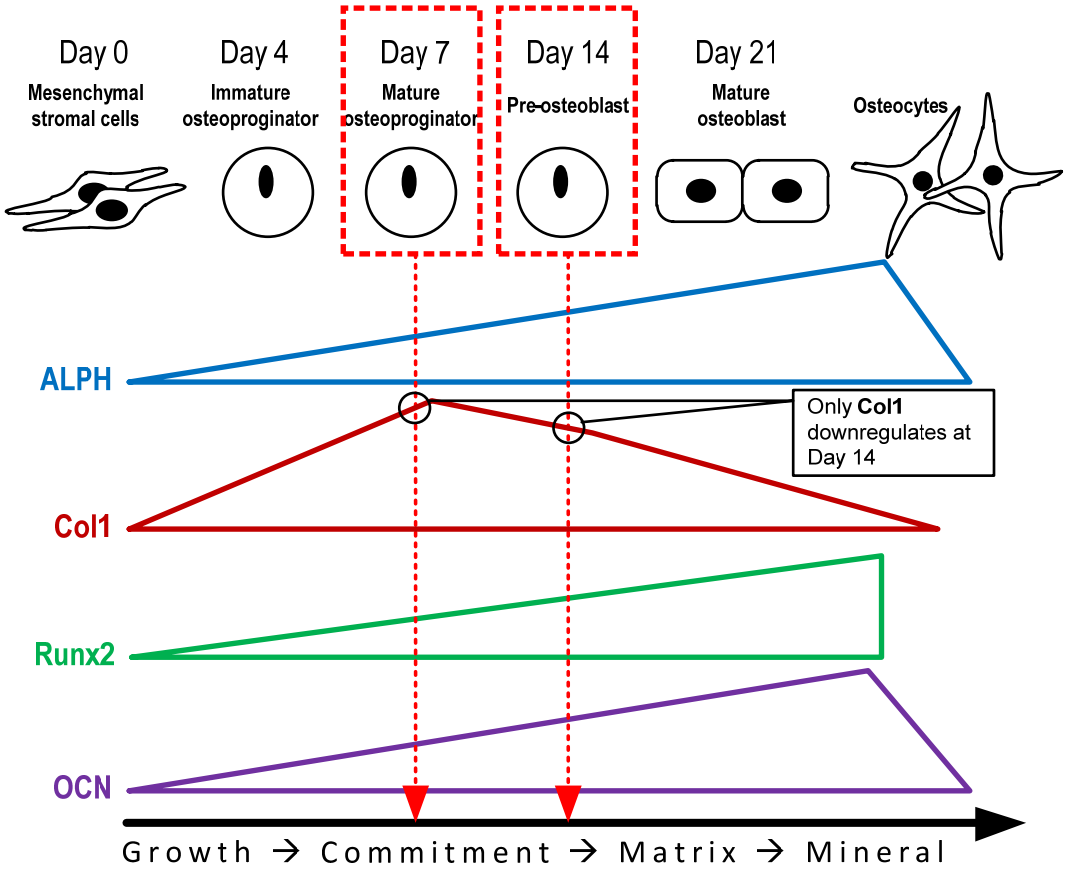

Figure 20. Temporal expression of markers during osteoblast differentiation in culture (adapted from Safadi et al 2009 [13]). While the other three markers increase gradually, Col-1 initially increases and decreases expression; we also observe this trend in **Figure 19**. However, we did not observe osteocalcin as it is usually detectable only in mature osteoblast (i.e. post-day 21). **Markers**: Runx2 = RUNX2; ALPH = alkaline phosphatase, Col-1 = Collagen type I, OCN = osteocalcin.

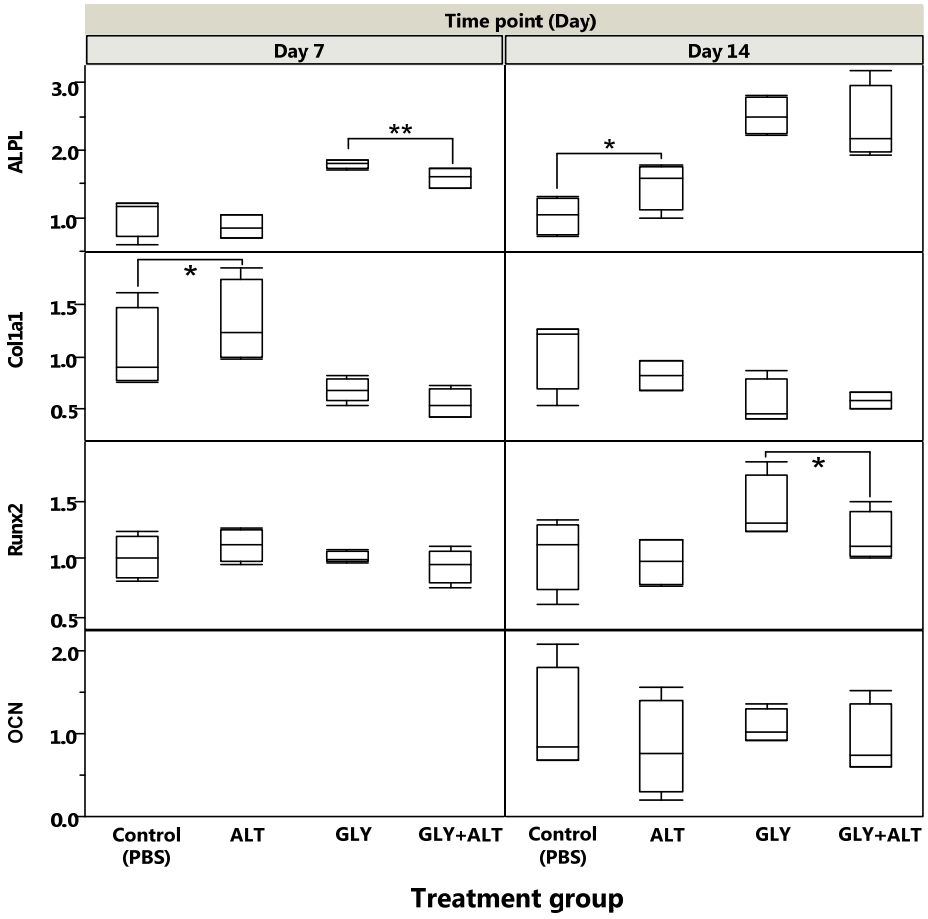

Figure 21. Boxplots of temporal osteogenic expressions grouped so that control (PBS) vs. ALT and GLY vs. GLY+ALT can be compared and potential effects of ALT on naturally (control) and artificially (GLY) glycated bone substrates can be determined. ALT was found to have significant up regulating effects on alkaline phosphatase (ALPH) at day 14 and collagen 1 (Col1a1) at day 7 in normal bone while it had down regulating effects on ALPH at day 7 and Runx2 at day 14 in artificially glycated bone. * = $p < 0.05$, ** = $p < 0.0001$

We found ALT treatment was associated with significant increases in collagen 1 (Col1a1) at day 7 and alkaline phosphatase (ALPH) at day 14 (**Figure 21**, $p = 0.0274$, $p = 0.048$ respectively), however ALT was associated with decreases in ALPH and Runx2 in the GLY+ALT group relative to GLY at day 14 ($p < 0.0001$, $p = 0.0492$ respectively). Although ALT's effect does not appear to be universal across all markers and GLY treatments, ALT at least partially promoted osteogenic expression in the bone substrates with naturally accumulated AGEs only (i.e. PBS-control) whereas it suppressed the expression markers of bone that has been artificially glycated (i.e. GLY). It is thus possible that ALT improves osteogenic activities in naturally aged bone. ALT can also suppress glycation effects on osteogenicity, consistent with the observation that it reduced AGE-fluorescence in glycated bone (**Figures 6-8**). However, it must be noted that the evidence of increased osteogenic activity when artificially glycated bone is used as substrate is in contrast to expectations from cell culture experiments that did not use bone substrates. It is possible that the type of AGE that ALT affected in naturally aged bone versus artificially aged bone could be different, however at this time we do not know why ALT have shown pro-osteogenic effects selectively. This increase of osteogenic activity with artificial glycation and its reversal with ALT requires further investigation.

In conclusion, the current stem cell culture protocol and RT-PCR method were able to replicate the temporal differentiation behavior of stem cells known from the literature. Furthermore our preliminary osteogenic data suggest that ALT can induce osteogenic effect on bone sections without any prior glycation treatment (i.e., naturally aged) and, in principal, support the basic idea of enhancing the osteogenic properties of a bone allograft using a crosslink breaker.

PROBLEMS ENCOUNTERED

The acquisition of cadaveric human tissues took considerably longer time than anticipated. This was despite initiating protocols with three tissue donation agencies. The original time estimate was based on previous experiences with the tissue banks; however due to a number of factors tissue acquisition has become more challenging in recent years. We have been able to acquire the approved amount of tissues and prepared them for the final tests before the end of the funding period. However, towards the end of the original funding period (2/19/2014), we were notified by the Headquarters, US Army Medical Research and Materiel Command (HQ USAMRMC) Office of Research Protections (ORP) that the Biological Resource Center (BRC) is under investigation by the Arizona Attorney General (AAG) and the FBI. We were instructed to halt research involving specimens from this cadaveric specimen supplier and quarantine the tissues until a decision is made.

Tissues acquired through one of the agencies (Platinum) turned out to be sourced through BRC and account for about 40% of our total cadaver material in this project. Even though we are prepared to move forward with these specimens, we have not received a decision from the ORP regarding the BRC specimens as of this writing.

We requested a no-cost extension in order to finish the work with the non-BRC specimens while providing additional time to work with the BRC specimens in case that a decision to proceed with them is made.

KEY RESEARCH ACCOMPLISHMENTS

We have procured and prepared 100% of femoral specimen requirement (24/24 femurs), obtaining all required CT specimens (n=96, mechanical test) and bone slices (n=500, stem cell experiment).

We have identified duration and concentrations required for ALT-711 (with and without ribose) that would result in detectable changes in the fluorescence levels in powdered bone and 2mm thick bone beams using both spectroscopy and/or epi-fluorescence microscopy.

We have completed glycation and ALT treatment of CT specimens and decalcified bone slices.

We have finalized fracture toughness test protocols and completed successful preliminary experiments and analysis on bovine CT specimens.

We have performed preliminary osteogenic expression tests on 60 bone sections that consistently replicated the known osteogenic behavior of bone cells on the specific substrates that are subject of this research.

We found evidences that ALT-711 can reduce levels of AGEs induced by ribose and improve osteogenic expression in untreated bone.

REPORTABLE OUTCOMES

No reportable outcome in terms of manuscripts, conference presentations or funding applied based on the work supported by this award is available at this time.

Employment or research opportunities applied for and/or received based on experience/training supported by this award

This project provided training opportunities for the following individuals whether or not they were compensated by the project funds:

1. Woong Kim, PhD: University of Auckland, New Zealand, Post-doc fellow, 2012 – present.
   RT-PCR analysis, sample procurement and preparation, fluorescence microscopy and fluorescence quantification, glycation+ALT-711 treatment, fracture toughness analysis.

2. Richard Banglmaier, PhD: Wayne State University, Senior Research Engineer, 2012-2013.
   Fluorescence quantification using spectrophotometry

3. Daniel Oravec, MSc: Tampere University of Technology, Finland, Research Engineer, 2012 – present
   Specimen procurement, accounting, machining and mechanical test system.

CONCLUSION

Currently allograft is the only practical source of bone grafts but it carries a plethora of issues such as nonunion due to poor bone quality as they are often sourced from old donors. One of the main contributing factors for poor bone quality is a natural accumulation of AGEs (advanced glycation endproducts) which forms non-enzymatic crosslinks that results in a brittle and biologically poor bones. It is proposed that once the crosslinks can be broken artificially by ALT-711, this will improve mechanical properties and create favorable bone matrix for the stem cells that will eventually result in a better integration in the patients.

In the past period, we have laid foundational work on the project - procuring femurs, refining and making bone samples, and optimizing treatment solution concentrations and durations. Furthermore, we have obtained preliminary evidence that ALT-711 can reduce level of AGEs induced by artificial glycation and induce positive osteogenic expressions in the osteoblastic stem cell culture. In the following months, we plan to complete the rest of prerequisite tasks for the main mechanical and biological tests, gather data and publish our findings.

REFERENCES

1. Wu, Z., A.J. Laneve, and G.L. Niebur, In vivo microdamage is an indicator of susceptibility to initiation and propagation of microdamage in human femoral trabecular bone. Bone, 2013. 55(1): p. 208-15.

2. Gustafson, M.B., et al., Calcium buffering is required to maintain bone stiffness in saline solution. J Biomech, 1996. 29(9): p. 1191-4.

3. Willett, T. L., S. Sutty, et al. (2013). "In vitro non-enzymatic ribation reduces post-yield strain accommodation in cortical bone." Bone **52**(2): 611-622.

4. Karim, L., S. Y. Tang, et al. (2013). "Differences in non-enzymatic glycation and collagen cross-links between human cortical and cancellous bone." Osteoporosis Int **24**(9): 2441-2447.

5. Brown, C. U., Yeni, Y. N. and Norman, T. L. (2000). “Fracture toughness is dependent on bone location--a study of the femoral neck, femoral shaft, and the tibial shaft.” J Biomed Mater Res 49(3): 380-389.

6. Yeni, Y. N., Brown, C. U., Wang, Z. and Norman, T. L. (1997). “The influence of bone morphology on fracture toughness of the human femur and tibia.” Bone 21(5): 453-459.

7. ASTM (1997). Standard Test Method for Plane-Strain Fracture Toughness of Metallic Materials. West Conshohocken, PA, American Society for Testing and Materials.

8. Saxena A, Hudak Jr SJ. (1978) Review and extension of compliance information for common crack growth specimens. Int J Fract.14:453–68

9. Nalla, R. K., J. J. Kruzic, et al. (2004). "On the origin of the toughness of mineralized tissue: microcracking or crack bridging?" Bone 34(5): 790-798.

10. Malik, C. L., S. M. Stover, et al. (2003). "Equine cortical bone exhibits rising R-curve fracture mechanics." J Biomech 36(2): 191-198.

11. Lian, J.B. and G.S. Stein, Development of the osteoblast phenotype: molecular mechanisms mediating osteoblast growth and differentiation. Iowa Orthop J, 1995. 15: p. 118-40.

12. Lian, J.B., et al., Regulatory controls for osteoblast growth and differentiation: role of Runx/Cbfa/AML factors. Crit Rev Eukaryot Gene Expr, 2004. 14(1-2): p. 1-41.

13. Safadi, F.F., et al., Bone Structure, Development and Bone Biology, in Bone Pathology, J.S. Khurana, Editor 2009, Humana Press: New York. p. 1-50.

APPENDICES

No appendix material is included.